Cell, Vol. 68, 143–155, January 10, 1992.

In Vivo Transfer of the Human Cystic Fibrosis Transmembrane Conductance Regulator Gene to the Airway Epithelium

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Summary

Direct transfer of the normal cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene to airway epithelium was evaluated using a replication-deficient recombinant adenovirus (Ad) vector containing normal human CFTR cDNA (Ad-CFTR). In vitro Ad-CFTR-infected CFPAC-1 CF epithelial cells expressed human CFTR mRNA and protein and demonstrated correction of defective cAMP-mediated Cl^- permeability. Two days after in vivo intratracheal introduction of Ad-CFTR in cotton rats, in situ analysis demonstrated human CFTR gene expression in lung epithelium. PCR amplification of reverse transcribed lung RNA demonstrated human CFTR transcripts derived from Ad-CFTR, and Northern analysis of lung RNA revealed human CFTR transcripts for up to 6 weeks. Human CFTR protein was detected in epithelial cells using anti-human CFTR antibody 11–14 days after infection. While the safety and effectiveness remain to be demonstrated, these observations suggest the feasibility of in vivo CFTR gene transfer as therapy for the pulmonary manifestations of CF.

Introduction

The clinical manifestations of cystic fibrosis (CF), a common lethal recessive hereditary disorder, are dominated by abnormalities of the airway epithelial surface, including chronic mucus production, infection, and inflammation (Boat et al., 1989). The gene responsible for CF, termed the cystic fibrosis transmembrane conductance regulator (CFTR) gene, is localized on chromosome 7 at q31 (Rommens et al., 1989; Riordan et al., 1989). The predicted CFTR protein is a 1480 residue glycosylated molecule with 12 transmembrane domains and 3 intracytoplasmic domains containing sequences that can be phosphorylated by protein kinases (Riordan et al., 1989; Gregory et al., 1990; Cheng et al., 1991; Bear et al., 1991). In vitro studies suggest that the CFTR protein is a Cl^- channel that modulates the permeability of Cl^- in response to elevations of intracellular cAMP (Anderson et al., 1991; Kartner et al., 1991; Bear et al., 1991). Mutations of the CFTR gene render epithelial cells unable to modulate Cl^- permeability through the cAMP pathway (Frizzell et al., 1986; Li et al., 1988; Hwang et al., 1989). Importantly, in vitro studies have shown that transfer of the normal CFTR cDNA to epithelial cell lines derived from individuals with CF can override this abnormality and permit the cells to secrete Cl^- in response to increased intracellular cAMP (Drumm et al., 1990; Rich et al., 1990).

These in vitro studies, and the knowledge that the lethal consequences of mutations of the gene occur almost exclusively in the lung (Boat et al., 1989), suggest the feasibility of somatic gene therapy for CF, i.e., it may be possible to correct the pulmonary manifestations caused by mutations of the CFTR gene by directly transferring a normal CFTR cDNA to airway epithelial cells in vivo. The major obstacles to this approach lie in the geometry of the lung and the biology of the airway epithelium. The epithelial cells of the human airway comprise an approximately 1–2 m^2 surface distributed over a successively branching "fractal-like" tree structure (Weibel, 1991), a geometry that makes it essentially impossible to treat CF successfully by removing the epithelial cells for in vitro correction and subsequent reimplantation. Furthermore, the majority of the airway epithelial cells are terminally differentiated, and those that are capable of proliferating do so at a slow rate (Evans and Shami, 1989). In view of these considerations, it is likely that somatic gene therapy for the respiratory manifestations of CF will require a methodology capable of transferring the normal cDNA into nonproliferating epithelial cells, and this will have to be done in vivo.

One possible solution to these problems is to utilize a replication-deficient recombinant adenovirus that contains an active promoter and a normal CFTR cDNA. In addition to the ability to accommodate a large (up to 7.5 kb) exogenous cDNA, the adenovirus has the advantages of being tropic for respiratory epithelium and capable of transferring recombinant genes into nonproliferating cells (Straus, 1984; Haj-Ahmad and Graham, 1986; Berkner, 1988; Horwitz, 1990). The recombinant adenoviral approach has been successful in transferring the human α1-antitrypsin gene to the respiratory epithelium of experimental animals in vivo (Rosenfeld et al., 1991a). The present study, utilizing a recombinant adenovirus containing a normal CFTR cDNA, demonstrates in vivo transfer and expression of the human CFTR gene to the respiratory epithelium of the lungs of cotton rats.

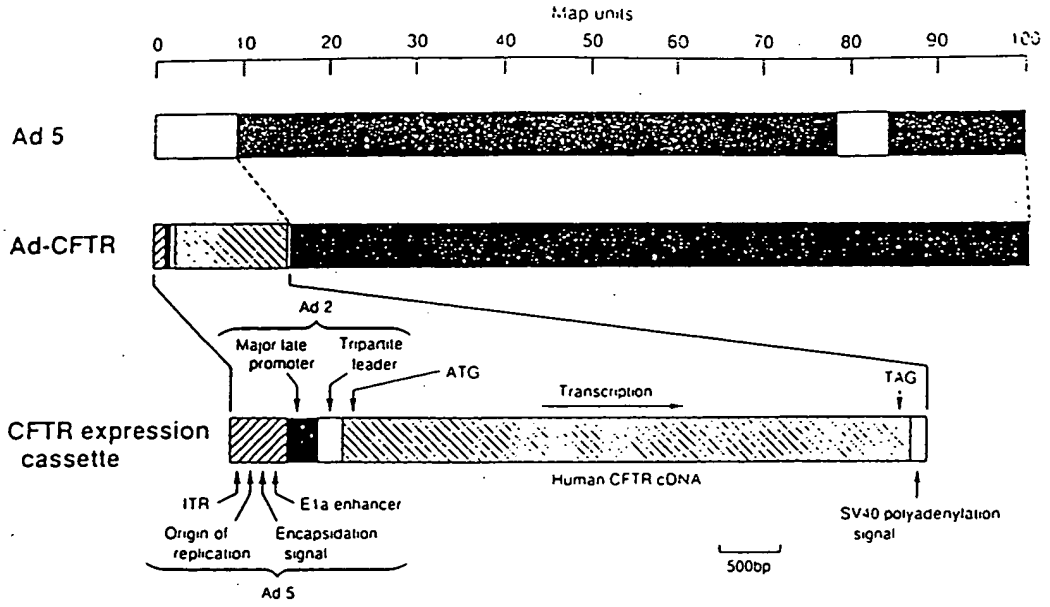

Figure 1. Schematic of the Recombinant Adenoviral Vector Ad-CFTR

Shown are the adenovirus type 5 (Ad 5) genome, the recombinant adenoviral DNA containing the human CFTR cDNA (Ad-CFTR), and a detailed enlargement of the CFTR expression cassette. Ad 5 DNA is divided into 100 mu (360 bp/mu). Stippled segments of Ad 5 indicate deletions of the left end of the genome, including the majority of the E1 region (0–9.2 mu) and E3 (78.4–84.3 mu), which were removed in the construction of Ad-CFTR to allow room for insertion of exogenous, nonviral DNA. The CFTR expression cassette includes the 5′ inverted terminal repeat (ITR), origin of replication, encapsidation signal, and E1a enhancer (all from Ad 5); the major late promoter and a copy of the tripartite leader sequence cDNA (both from Ad 2); the entire 4.5 kb protein coding sequence of the human CFTR cDNA; and the SV40 early mRNA polyadenylation signal. The CFTR protein translation start (ATG) and stop (TAG) signals are indicated.

Results

Construction of the Recombinant Adenoviral Vector Ad-CFTR

Ad-CFTR, a replication-deficient recombinant adenovirus containing the human CFTR protein coding sequence cDNA, was constructed using a modified type 5 adenoviral (Ad 5) genome (Figure 1). The majority of the E3 region (78.4–84.3 map units [mu]) was deleted to provide space for insertion of a cassette containing the 4.5 kb coding sequence of the human CFTR cDNA (Thimmappaya et al., 1982; Riordan et al., 1989). The left end of the viral genome (0–9.2 mu, including the E1a and the majority of the E1b region) was deleted and replaced by a CFTR expression cassette containing essential viral cis-acting elements, including the inverted terminal repeat (ITR), origin of replication, and the encapsidation signal, as well as the E1a enhancer, but not the E1a structural gene, a substitution that eliminates autonomous replication of the recombinant vector by removal of the E1a function. The E1a enhancer was followed by the adenovirus type 2 (Ad 2) major late promoter (MLP) and a 4.5 kb CFTR cDNA (nucleotides 123–4587; see Riordan et al. [1989] for sequence numbering). The Ad 2 MLP was used to drive transcription of human CFTR cDNA sequences. The majority of the Ad 2 tripartite leader sequence cDNA was included to increase the translation efficiency of expression of CFTR protein (Manseur et al., 1988). Ad-CFTR was replicated in the permissive cell line 293 (a human kidney cell line containing a function in E1a gene that provides a trans-acting E1a protein [Graham et al., 1977]) and high titer, infectious Ad-CFTR viral stocks were prepared.

In Vitro Evaluation of Ad-CFTR-Directed Human CFTR mRNA Transcripts

Ad-CFTR directed the expression of human CFTR mRNA transcripts in vitro (Figure 2). As previously observed, Northern analysis of T84 human colon carcinoma cells (lane 1) demonstrated a 6.5 kb CFTR mRNA transcript (Riordan et al., 1989; Drumm et al., 1990; Kartner et al., 1991; Yoshimura et al., 1991a; Trapnell et al., 1991a), while uninfected 293 or CFPAC-1 cells did not contain CFTR mRNA transcripts detectable by Northern analysis using the 4.5 kb human CFTR probe (lanes 2 and 5, respectively). Similarly, in vitro infection of 293 cells (lane 3) or CFPAC-1 cells (lane 6) by the control virus Ad-dl312 (Ad 5 with a deletion of the E1a region [1.5–4.5 mu; Jones and Shenk, 1979]), which does not contain the CFTR cDNA, did not demonstrate detectable CFTR transcripts. In contrast, a 5.2 kb transcript (the expected size of Ad-CFTR-directed human CFTR mRNA transcripts) was observed after in vitro Ad-CFTR infection of 293 cells (lane 4) or CFPAC-1 cells (lane 7). The levels of human β-actin transcripts were similar in all samples (data not shown).

In Vitro Function of the Ad-CFTR-Directed Human CFTR Protein

As there is no animal model for cystic fibrosis, it is not possible to demonstrate definitively the function of the Ad-CFTR-directed CFTR protein in vivo. However, the

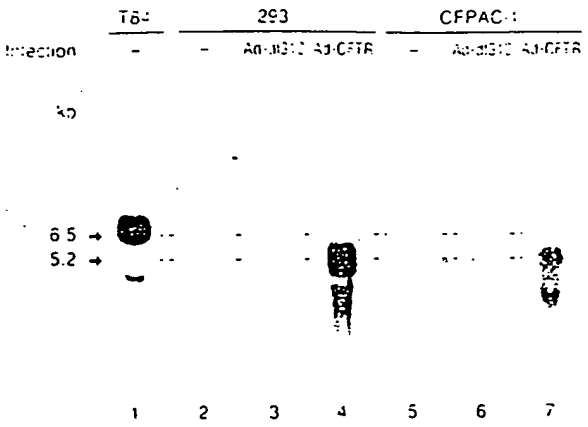

Figure 2. Evaluation of Human CFTR mRNA Transcripts after In Vitro Ad-CFTR Infection

Shown are Northern analyses of total cellular RNA (10 μg per lane except for 293 cells, 5 μg per lane) evaluated with a 4.5 kb human CFTR cDNA probe. Lane 1: uninfected T84 cells; lane 2: uninfected 293 cells; lane 3: 293 cells infected with Ad-dl312; lane 4: 293 cells infected with Ad-CFTR; lane 5: uninfected CFPAC-1 cells; lane 6: CFPAC-1 cells infected with Ad-dl312; lane 7: CFPAC-1 cells infected with Ad-CFTR. The 6.5 kb endogenous human CFTR mRNA transcript is indicated, as is the 5.2 kb Ad-CFTR-directed CFTR mRNA transcript. The latter is expected to be smaller than the endogenous human cell transcript, because of the deletion of 5' and 3' untranslated sequences from the CFTR cDNA in the construction of Ad-CFTR (see Figure 1).

Ad-CFTR vector clearly directed the biosynthesis of functional CFTR protein as demonstrated by in vitro studies in cultured cells (Figure 3). In this regard, in cells modified in vitro by Ad-CFTR infection, the de novo biosynthesis of human CFTR protein was evaluated using metabolic labeling and immunoprecipitation of the CFTR protein with a mouse anti-human CFTR-specific monoclonal antibody. Evaluation of Ad-CFTR-infected 293 cells demonstrated the presence of a new 165 kd protein and a minor 141 kd protein (Figure 3A, lane 2), neither of which was detectable in uninfected cells (lane 1). CFPAC-1 cells infected by Ad-CFTR also showed a new protein of 165 kd (Figure 3, lane 4) not present in Ad-α1AT-infected (lane 3) or uninfected cells (data not shown). For both 293 and CFPAC-1 cells, the size of the major Ad-CFTR-directed protein (165 kd) is within the range of the size detected in T84 cells (data not shown) and that expected for a completely processed form of the glycosylated protein (Riordan et al., 1989; Cheng et al., 1990; Kartner et al., 1991).

The ability of Ad-CFTR to impart cAMP-regulated Cl^- permeability to cells that do not normally exhibit this physiologic function (293 cells) and to correct the defective cAMP-stimulated up-regulation of Cl^- permeability in epithelial cells derived from individuals with CF (CFPAC-1 cells) was evaluated by examining forskolin-stimulated $^{36}Cl^-$ efflux (Figures 3B–3F). Consistent with the lack of detectable endogenous CFTR mRNA expression by Northern analysis, uninfected 293 cells lacked the ability to up-regulate Cl^- permeability in the presence of forskolin (Figure 3E). In contrast, after Ad-CFTR infection, forskolin significantly stimulated Cl^- permeability (Figure 3C). As [illegible] CFPAC-1 cells (Figure 3D) or CFPAC-1 cells infected with the control virus Ad-α1AT (3E) did not demonstrate forskolin-stimulated Cl^- permeability. However, after in vitro infection with Ad-CFTR, CFPAC-1 cells exhibited a significant increase in forskolin-stimulated Cl^- permeability (Figure 3F), indicating correction of the CF epithelial cell phenotype and, thus, the function of the Ad-CFTR-directed product. Of note is the small increase in basal $^{36}Cl^-$ efflux in both cell lines only when infected with Ad-CFTR (but not Ad-α1AT, an irrelevant cDNA virus control). One possible explanation for this phenomenon is that these cells express more CFTR protein than uninfected cells, and their basal Cl^- permeability may be increased due to an increase in the absolute number of Cl^- channels.

Expression of Ad-CFTR-Directed Human CFTR mRNA in Bronchial Epithelium In Vivo

Following intratracheal administration of Ad-CFTR in cotton rats in vivo, the presence of human CFTR mRNA transcripts could be detected in bronchial epithelium by in situ hybridization analysis using a ^{35}S-labeled human CFTR cRNA probe (Figure 4). Control animals not infected with Ad-CFTR did not show hybridization with the antisense probe (Figure 4A). In contrast, hybridization of the antisense probe demonstrated expression of human CFTR mRNA sequences diffusely throughout the airway epithelium and subepithelium of animals infected with Ad-CFTR (Figures 4C, 4E, and 4G). As a further control, the sense probe did not show hybridization in bronchial tissues from either uninfected (Figure 4B) or Ad-CFTR-infected cotton rats (4D, 4F, and 4H). The absence of hybridization with the sense probe in the lung of Ad-CFTR-infected animals also indicated that residual Ad-CFTR DNA (which might theoretically be present in the tissue) was not detectable. Quantitative evaluation of the expression of human CFTR mRNA in the epithelium, compared with the subepithelium after adenovirus-mediated human CFTR gene transfer, demonstrated that the average signal over the epithelium obtained with the antisense probe was 1.6 times that over the subepithelium ($p = 0.025$). (All statistical comparisons were made using the two-tailed Student's t-test.)

Specificity and Chronicity of Expression of Human CFTR mRNA Transcripts Following In Vivo Infection

To further confirm the detection of human CFTR mRNA transcripts in the lungs of cotton rats infected with Ad-CFTR, a strategy was employed which allows detection of only those transcripts with contiguous viral and human RNA sequences expected to be directed by Ad-CFTR. This was accomplished by amplification of Ad-CFTR mRNA (after conversion to cDNA) using the polymerase chain reaction (PCR) with two pairs of primers: an adenoviral sense primer for viral sequences 5' to the viral/human CFTR junctional sequences and an opposing human CFTR cDNA antisense primer located within human CFTR exon 5, and a human CFTR cDNA sense primer located within exon 21 and a viral antisense primer for [illegible] sequences 3' to the human/viral RNA junctional sequences (Figures 1 and 5A). To ensure that

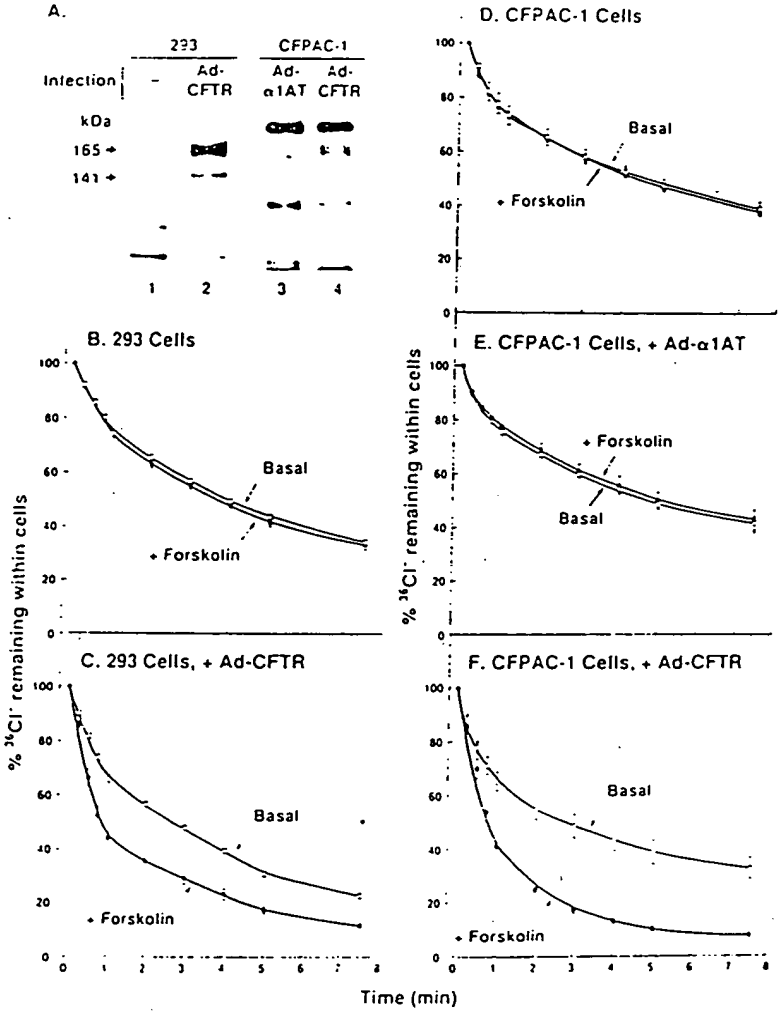

Figure 3. In Vitro Evaluation of the Form and Function of the Human CFTR Protein Directed by Ad-CFTR

(A) De novo biosynthesis of human CFTR. Cells were infected, labeled with [^{35}S]methionine, and evaluated for ^{35}S-labeled CFTR by immunoprecipitation as described in Experimental Procedures. Lane 1: uninfected 293 cells; lane 2: 293 cells after infection with Ad-CFTR; lane 3: CFPAC-1 cells infected with the control virus Ad-α1AT; lane 4: CFPAC-1 cells infected with Ad-CFTR. The major 165 kd CFTR protein and minor 141 kd bands are indicated.

(B–F) Evaluation of the functional ability of Ad-CFTR-derived human CFTR to modulate forskolin-stimulated Cl^- permeability. $^{36}Cl^-$ efflux was evaluated at rest (basal) and after stimulation (forskolin) as described in Experimental Procedures. The data are presented as a percentage of Cl^- remaining in cells at each time point under basal or forskolin-stimulated conditions; each data point represents the mean of separate determinations (for each data point in B, C, and E, $n = 3$; in D and F, $n = 4$). Evaluation of forskolin-stimulated Cl^- efflux in 293 human embryonic kidney cells before and after infection with Ad-CFTR is shown for uninfected 293 cells (B) and 293 cells infected with Ad-CFTR (C). Ad-CFTR correction of the Cl^- efflux defect in epithelial cells derived from an individual homozygous for the ΔF508 CFTR mutation is shown for uninfected CFPAC-1 cells (D), CFPAC-1 cells infected with Ad-α1AT, as a negative control (E), and CFPAC-1 cells infected with Ad-CFTR (F).

sequences were being detected and to exclude detection of potentially contaminating Ad-CFTR DNA, RNA samples were exposed to DNAase I prior to conversion of purified RNA to cDNA and subsequent PCR. Furthermore, each sample was evaluated in the absence or presence of reverse transcriptase. Ad-CFTR-directed human CFTR transcripts were detected using the 5′ region primer pair after Ad-CFTR infection, but not in uninfected animals or after Ad-dl312 infection (Figure 5B). In the absence of reverse transcriptase, none of the samples amplified CFTR mRNA sequences. As a control, rat glyceraldehyde-3-phosphate dehydrogenase mRNA transcripts were detected in rat lung total RNA in all samples after incubation with reverse transcriptase, but not in the absence of reverse transcriptase (Figure 5C).

The chronicity of Ad-CFTR-directed CFTR mRNA expression was demonstrated in cotton rat lung evaluated 2, 4, and 6 weeks after in vivo infection with Ad-CFTR. Ad-CFTR-directed transcripts were observed at all times with the 5′ region amplification (Figure 5D) and also with the 3′ region amplification (5E). In the absence of reverse transcriptase, no sample showed amplification of CFTR [illegible].

Consistent with the PCR evaluation, Northern analysis of lung RNA showed Ad-CFTR-directed human CFTR mRNA transcripts of a size similar to that directed by Ad-CFTR in cultured cells (Figure 2, lanes 4 and 7), in total lung RNA at 2 days (Figure 6, lane 3) and up to 42 days (Figure 6, lane 4) after in vivo infection of cotton rats by intratracheal instillation of Ad-CFTR, but not in uninfected animals (Figure 6, lane 2). Quantitative evaluation of the level of human CFTR expression demonstrated that the signal intensity at 42 days was 42% of that at 2 days. After very long exposure times, transcripts larger than Ad-CFTR-derived transcripts were detectable in uninfected and Ad-dl312-infected animals; this likely represents the cotton rat endogenous CFTR transcripts (data not shown). Levels of rat glyceraldehyde-3-phosphate dehydrogenase transcripts were similar for all lung samples (data not shown).

Detection of Human CFTR Protein after In Vivo Adenovirus-Mediated Gene Transfer

Immunohistochemical evaluation with an anti-human CFTR antibody demonstrated human CFTR protein in cotton rat respiratory epithelial cells following in vivo [illegible]

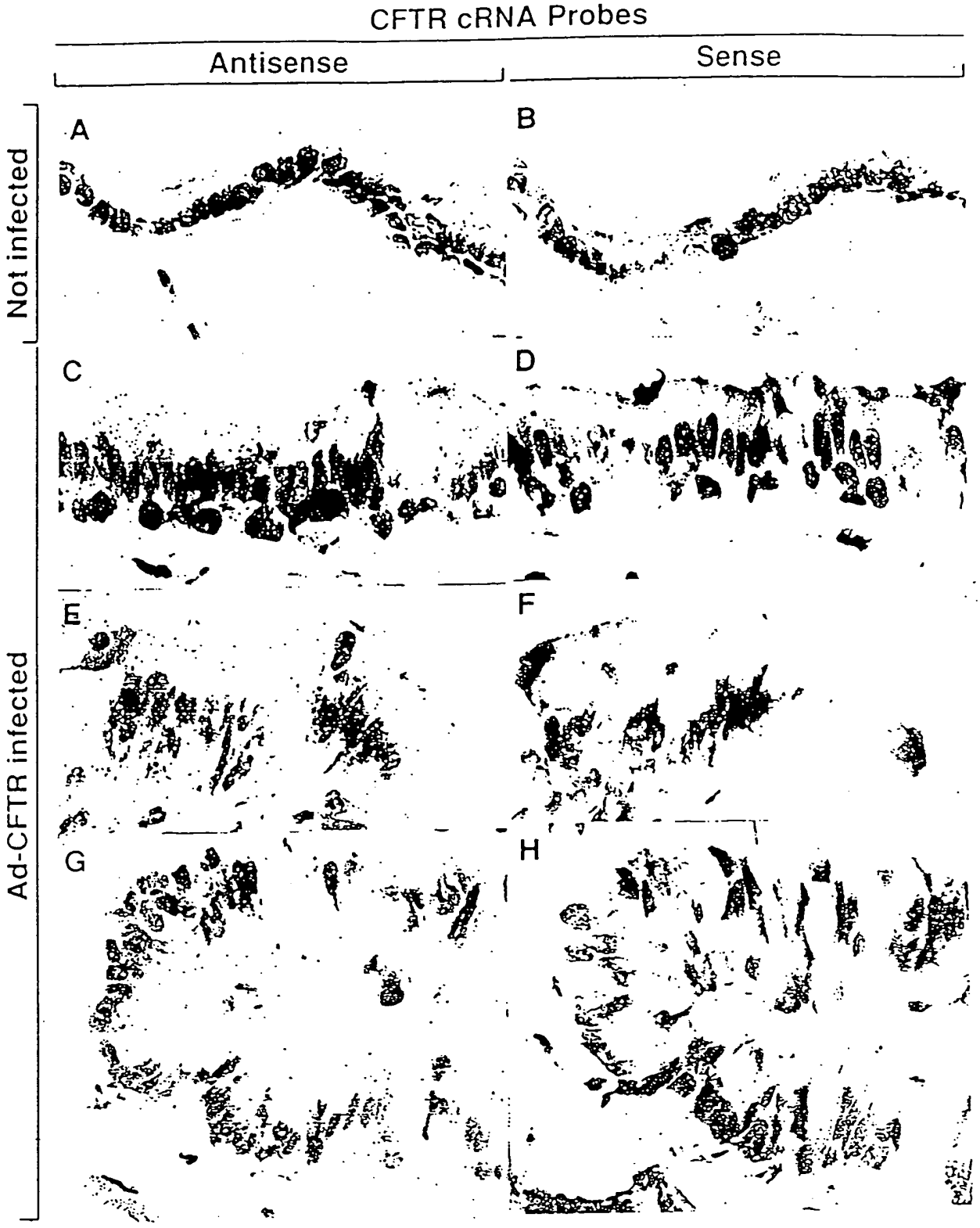

Figure 4. Evaluation of Ad-CFTR-Directed Human CFTR mRNA Transcripts in Lungs of Cotton Rats by In Situ Hybridization Analysis

Shown are sections through bronchial epithelium from an uninfected animal and an animal 2 days after infection with Ad-CFTR, evaluated with antisense CFTR cRNA probe (A, C, E, and G) and control, a sense CFTR probe (B, D, F, and H). (A and B) Uninfected cotton rat. (C–H) Cotton rat infected with Ad-CFTR.

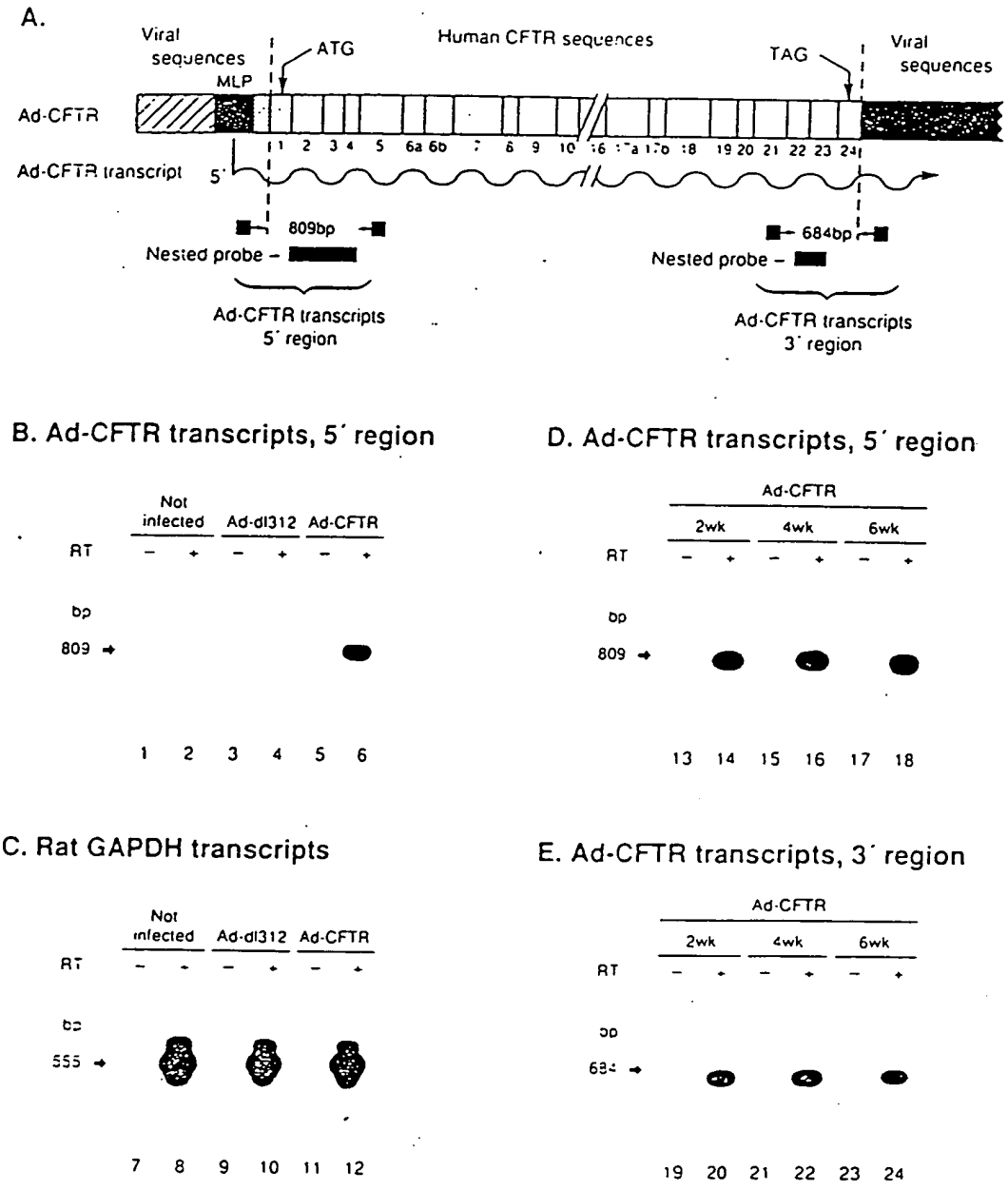

Figure 5. Evaluation of the Specificity and Chronicity of Human CFTR Gene Expression in Cotton Rat Lung Following In Vivo Infection with Ad-CFTR

(A) Schematic of a portion of Ad-CFTR showing the Ad-CFTR-derived CFTR mRNA transcript and the location of primer pairs used to identify the Ad-CFTR-directed mRNA transcripts specifically. Ad-CFTR DNA sequences shown include (from left): adenoviral expression cassette sequences, including the major late promoter (MLP), the human CFTR cDNA coding sequence (CFTR exons are indicated by numbers below Ad-CFTR), and the remainder of the adenoviral vector genome. The Ad-CFTR transcript 5' amplification primer pair consists of a 5' viral-specific sense primer and a 3' human CFTR cDNA-specific antisense primer. The 3' primer pair consists of a 5' human CFTR cDNA-specific sense primer and a 3' viral-specific antisense primer. Also shown are the sizes of the expected amplification products and the "nested" probes used to detect specifically amplified Ad-CFTR transcripts. mRNA was converted to cDNA and amplified as described in Experimental Procedures.

(B–E) mRNA transcripts in cotton rat lung following in vivo infection with Ad-CFTR. "–" and "+" indicate the absence and presence of reverse transcriptase (RT), respectively, in cDNA synthesis reactions. The sizes of expected amplification products are indicated in each panel. (B) Evaluation of the 5' region of Ad-CFTR-directed mRNA transcripts. Lane 1: uninfected cotton rat lung RNA without RT; lane 2: with RT; lane 3: 2 days after infection with Ad-dl312, without RT; lane 4: with RT; lane 5: 2 days after infection with Ad-CFTR, without RT; lane 6: with RT. (C) Evaluation of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA transcripts. Lane 7: uninfected cotton rat lung RNA, without RT; lane 8: with RT; lane 9: 2 days after infection with Ad-dl312, without RT; lane 10: with RT; lane 11: 2 days after infection with Ad-CFTR, without RT; lane 12: with RT. (D) Evaluation of the 5' region of Ad-CFTR-derived CFTR mRNA transcripts in cotton rat lung after infection with Ad-CFTR. Lane 13: 2 weeks after infection, without RT; lane 14: with RT; lane 15: 4 weeks after infection, without RT; lane 16: with RT; lane 17: 6 weeks after infection, without RT; lane 18: with RT. (E) 3' region amplification of CFTR in cotton rat lung after infection with Ad-CFTR. Lane 19: 2 weeks after infection, without RT; lane 20: with RT; lane 21: 4 weeks after infection, without RT; lane 22: with RT; lane 23: 6 weeks after infection, without RT; lane 24: with RT.

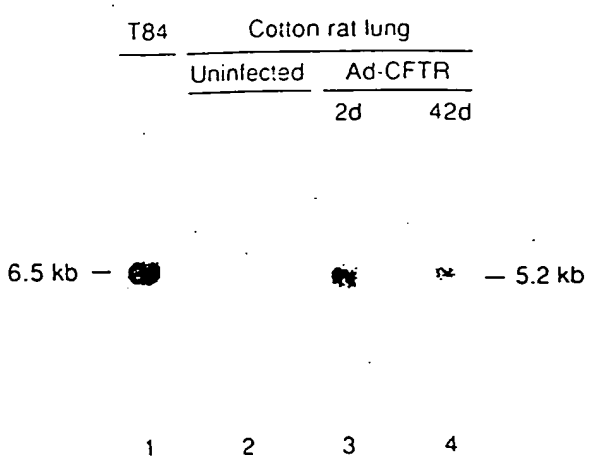

Figure 6. Evaluation of Human CFTR mRNA Transcripts after In Vivo Ad-CFTR Infection of Cotton Rat Lung

Shown is a Northern analysis of total cellular RNA from cotton rat lung (10 μg per lane) evaluated with a 4.5 kb human CFTR probe. Lane 1: uninfected T84 cells as a positive control; lane 2: uninfected cotton rat lung; lane 3: cotton rat lung 2 days after infection with Ad-CFTR; lane 4: cotton rat lung 42 days after infection with Ad-CFTR. The 6.5 kb endogenous human CFTR mRNA transcript is indicated (lane 1), as is the 5.2 kb Ad-CFTR-directed CFTR mRNA transcript (lanes 3 and 4).

cheal installation of Ad-CFTR (Figure 7). As a control, the anti-CFTR antibody demonstrated human CFTR protein in T84 cells (a cell line known to express CFTR; Figure 7A). Human CFTR was not found in uninfected cotton rat respiratory epithelial cells evaluated in vitro (Figure 7B), but was present in cotton rat respiratory epithelial cells infected in vitro with Ad-CFTR (7C). Human CFTR protein was not detected in cotton rat respiratory epithelium of uninfected animals (Figures 7D and 7E) or animals infected with the control virus Ad-dl312 (7F). Importantly, human CFTR protein was detected in cotton rat respiratory epithelial cells 11–14 days after in vivo infection with Ad-CFTR (Figures 7G–7K), but not when the primary antibody was omitted (7L) or when the primary antibody was replaced by an irrelevant antibody of the same antibody subclass (data not shown).

Discussion

This study presents a strategy for gene therapy for the respiratory manifestations of CF using the direct transfer of the normal human CFTR cDNA to the respiratory epithelium in vivo utilizing a recombinant, replication-deficient adenoviral-based vector. The rationale of this approach is based on several facts, including the following: the lethal manifestations of CF involve the respiratory epithelium (Boat et al., 1989), the CFTR gene is expressed in airway epithelial cells (Jefferson et al., 1990; Yoshimura et al., 1991a; Chu et al., 1991; Trapnell et al., 1991b), the respiratory manifestations of CF do not recur following transplantation of a normal lung into a CF individual (de Leval et al., 1991), the defective cAMP-mediated Cl^- permeability in epithelial cells derived from individuals with CF can be restored by transfer of a normal human CFTR cDNA into the cells (Drumm et al., 1990; Rich et al., 1990), the complex anatomy of the human airway epithelial surface precludes the approach to gene therapy of removing defective cells for in vitro gene transfer of a normal gene and reimplantation of these cells (Weibel, 1991), and the slow rate of respiratory tract epithelial cell turnover favors a vector (such as adenovirus) that does not require host cell proliferation for recombinant gene expression (Berkner, 1988; Evans and Shami, 1989).

Several observations in the present study suggest that this approach is feasible. In vitro studies demonstrated that Ad-CFTR was capable of directing the synthesis of intact, functional CFTR protein. In this regard, in human embryonic kidney 293 cells (cells that normally do not express detectable CFTR mRNA transcripts by Northern analysis nor demonstrate cAMP-mediated Cl^- permeability as evidenced by $^{36}Cl^-$ efflux studies) and in CFPAC-1 cells (a human pancreatic epithelial cell line derived from a ΔF508 CF homozygote that expresses CFTR mRNA at low levels detectable by PCR and expresses the "CF phenotype," i.e., does not secrete Cl^- in response to cAMP [Schoumacher et al., 1990]), infection with Ad-CFTR resulted in detectable de novo biosynthesis of CFTR molecules and conveyed to these cells the ability to increase Cl^- permeability in response to elevations in cAMP. Following in vivo respiratory tract infection with Ad-CFTR, in situ hybridization analysis demonstrated Ad-CFTR-derived human CFTR mRNA expression in the respiratory epithelium, which is a site of the CF epithelial cell Cl^- secretory defect (Knowles et al., 1981, 1983; Frizzell et al., 1986; Li et al., 1988; Jetten et al., 1989; Boat et al., 1989; Hwang et al., 1989; Rich et al., 1990; Zeitlin et al., 1991) and where the CFTR gene is expressed (Yoshimura et al., 1991a; Chu et al., 1991; Trapnell et al., 1991b). Furthermore, human CFTR mRNA transcripts were detected in cotton rat lungs for up to 6 weeks. Finally and most importantly, human CFTR protein was detected in cotton rat respiratory epithelium after in vivo Ad-CFTR infection. The localization of the human CFTR appears to be primarily intracellular rather than on the cell surface. Additional studies will have to be carried out to determine the exact location of the Ad-CFTR-directed gene product.

Requirements for Ad-CFTR-Directed Human CFTR Gene Therapy

While the present study demonstrates that it is possible to use an adenoviral vector to transfer a recombinant normal CFTR cDNA to the lung, where it is expressed for at least 6 weeks, several questions have to be addressed in the context of applying this therapy to individuals with CF.

First, decisions will have to be made about the threshold levels of expression and the specific epithelial cell targets necessary to reverse the disease process. It is known that individuals heterozygous for the normal and common abnormal ΔF508 CFTR alleles are clinically well and express both alleles in the respiratory epithelium equally at the mRNA level (Trapnell et al., 1991b). Quantitative studies show that CFTR mRNA is expressed in low abundance at about 1–2 copies per cell on average in the human airway

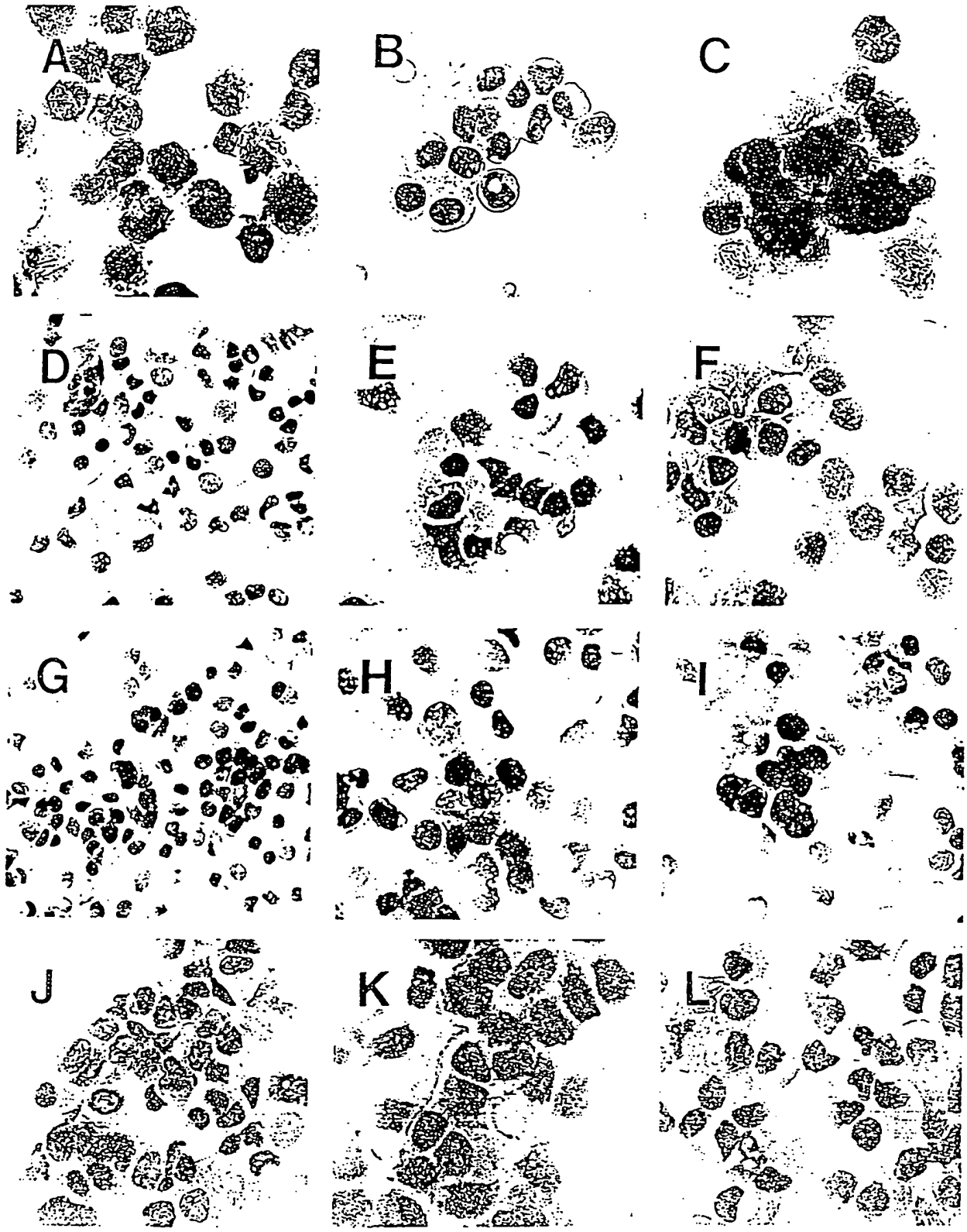

Figure 7. Immunohistochemical Localization of Human CFTR after In Vivo Infection of Cotton Rat Lung with Ad-CFTR

Shown are cytocentrifuge preparations of various controls and of cells obtained by cytologic brush from cotton rat respiratory tract obtained 11–14 days after Ad-CFTR intratracheal instillation. Immunoreactivity to human CFTR using a primary mouse anti-human CFTR monoclonal antibody is indicated by a red color, and cell nuclei appear blue as a result of hematoxylin counterstaining. (A) Control T84 colon epithelial cells (× 1000). (B) Cotton rat airway epithelial cells, uninfected (× 1000). (C) Same as (B), but infected in vitro with Ad-CFTR (× 1000). (D) Cotton rat airway epithelial cells from an uninfected animal, 14 days after instillation of the virus dialysis buffer (× 830). (E) Similar to (D), 11 days after receiving PBS (× 1000). (F) Cotton rat airway epithelial cells from an animal 11 days after infection with the control virus Ad-dl312 (× 1000). (G) Cotton rat airway epithelial cells from an animal 11 days after infection with Ad-CFTR (× 830). (H and I) Same as (F) but [illegible] (× 1000). (J) Similar to (G) but [illegible] (× 1000). (K) Same as (G) but [illegible] (× 1000). (L) Similar to (G) but [illegible] (× 1000).

epithelium, and indirect evidence suggests that the CFTR gene is likely expressed in the ciliated cell, the predominant cell type found in this epithelium (Trapnell et al., 1991b). Together, this evidence leads to the working hypothesis that gene therapy for CF will require only low-level expression of the normal CFTR to correct the defective physiology in the airway epithelium. Consistent with this concept, complementation studies in which the normal CFTR cDNA has been transferred with a retrovirus in vitro, to correct the abnormal CF Cl^- secretory phenotype in epithelial cells derived from an individual with CF, suggest that low-level expression of the normal CFTR gene is sufficient (Drumm et al., 1990). Consequently, the observations that Ad-CFTR-directed human CFTR mRNA transcripts can be detected by in situ hybridization, PCR, and Northern analysis in cotton rat lung after Ad-CFTR infection in vivo, and that human CFTR protein can be detected in airway epithelial cells recovered from these animals, suggest that this approach could provide adequate expression to complement the abnormal CFTR gene expression in respiratory epithelium of CF individuals. In regard to delivery to specific cell types, in situ hybridization analysis of Ad-CFTR-infected animals suggests that the majority of the Ad-CFTR expression in the airway is in the epithelium, consistent with the known site of expression of defective Cl^- permeability in CF. It is not clear which cells in the subepithelium are expressing the Ad-CFTR product. As the CFTR gene is expressed in a broad range of human cells (Yoshimura et al., 1991b), this could represent natural sites of rat lung expression (Tresize et al., 1991). The paucity of submucosal glands and the abundance of epithelial surface secretory cells in rodents compared with humans hinders a direct comparison between exogenous CFTR expression in these animals and the endogenous expression of CFTR in the lungs of normal humans.

Second, while low levels of expression of CFTR in most airway epithelial cells will likely correct the abnormal CF phenotype, the consequences of excess expression of the normal CFTR gene in these cells are unknown. In vitro, overexpression with a vaccinia vector corrects the CF Cl^- secretory phenotype in a fashion similar to that of low-level expression with retrovirus (Rich et al., 1990; Gregory et al., 1990), and as shown in the present study, similar to the level of expression achieved with the adenovirus vector.

Third, the safety of the viral vector system is important. In this regard, the Ad-CFTR recombinant adenoviral vector was constructed to be replication deficient by removal of E1a and the majority of E1b structural genes. As might be expected from the natural tropism of adenovirus for human respiratory epithelial cells, Ad-CFTR can readily infect human respiratory epithelial cells (Straus, 1984; Rosenfeld et al., 1991b), but once inside these cells, the vector should not replicate autonomously. This inability to replicate limits the recombinant adenoviral infection to those epithelial cells infected initially; in contrast, wild-type adenovirus is capable of lytic infection of host cells, which is characterized by viral self-replication and infection of surrounding cells. By rendering Ad-CFTR replication deficient, the most important negative consequence of adenoviral infection, cell death, should be eliminated and the biosynthetic machinery of the host cells should not be dominated by viral replication, as is the case with a replication-competent virus. One potential problem with recombinant vectors made from E1a deletion mutants is the possibility that the host cell could overcome their defective replication by providing the E1a function in trans, thus allowing viral replication, albeit at a lower level than seen with wild-type adenovirus. Alternatively, a concurrent wild-type adenovirus infection could result in recombinant adenoviral replication, either by complementation or by a recombination event.

Fourth, a rational strategy for gene therapy for CF requires consideration of whether the newly introduced CFTR gene will need to be regulated or whether constitutive expression will be sufficient. The structural features of the CFTR gene promoter place it in the housekeeping class, implying that its expression may be constitutive (Yoshimura et al., 1991a). However, the presence of multiple potential binding sites for known transcriptional regulatory factors suggest that it may also be regulatable, and modulation of CFTR gene expression has been demonstrated in vitro (Yoshimura et al., 1991a; Trapnell et al., 1991a). As these questions are yet unanswered, for now our current vectors utilize a constitutive promoter. However, if regulation of Ad-CFTR-directed CFTR expression is important, a vector with the CFTR promoter could be used.

Finally, the adenovirus has advantages as a vector system in gene therapy of CF with regard to respiratory epithelial tropism, ease of producing high titer recombinant virus, high infectivity rate, and no dependence on target cell proliferation. It is not known what proportion, if any, of the recombinant DNA is integrated into the genome of the target cells; thus the persistence of expression is unknown. However, because human CFTR transcripts can be detected in cotton rat lung for up to 6 weeks after a single instillation of Ad-CFTR, this may not be an important problem. Once the threshold level of CFTR expression necessary for normal function is known, if Ad-CFTR-directed expression is insufficient, barring safety problems regarding the antigenicity of the vector, the easy access of the airway epithelial surface should permit repetitive delivery.

Experimental Procedures

Cell Cultures

The transformed human embryonic kidney cell line 293 (American Type Culture Collection [ATCC] CRL 1573 [Graham et al., 1977]) was grown in Improved minimal essential medium (Biofluids) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. CFPAC-1 cells, originally derived from a pancreatic adenocarcinoma of an individual homozygous for the common ΔF508 CF mutation (Schoumacher et al., 1990), were grown in Dulbecco's modified Eagle's medium (DMEM, Biofluids) with supplements as above. The human colon adenocarcinoma cell line T84 (ATCC CCL 248) was cultured as for CFPAC-1 cells, except with 5% FBS.

Construction of the Recombinant Adenoviral Vector Ad-CFTR

The recombinant adenovirus Ad-CFTR was constructed from human adenovirus type 5 (Ad 5) deletion mutant, Ad-dl324 (Thimmappaya et al., 1982), and a plasmid (pTG5955) containing the 5′ [illegible] terminal origin of replication, encapsidation signal, and 5′

(all from Ad 5); the major late promoter and the majority of the tripartite leader sequence cDNA (both from Ad 2); the 4.5 kb human CFTR cDNA, including the entire protein coding sequence (from nucleotide 123 to 4587; see Riordan et al., 1989, for sequence numbering) with minor sequence differences, which include a silent mutation (T→C at nucleotide position 1227), a known polymorphism (A[Met]→G[Val] at nucleotide position 1540 [Kerem et al., 1990]), and two changes from the original description of the sequence (A→C at nucleotide position 1990 and C→T at nucleotide position 2629 [Riordan et al., 1989]) produced from oligo(dT)-primed cDNA and PCR-amplified cDNA fragments derived from human lung poly(A)$^+$ RNA, and propagated without rearrangement in the E. coli strain BJ5183 (Hanahan, 1983); the SV40 early polyadenylation signal; and the Ad 5 sequences from nucleotide positions 3329–6241. pTG5955 was linearized by ClaI cleavage and cotransfected with the large fragment of ClaI-cut Ad-dl324 DNA into 293 cells to allow homologous recombination to occur, followed by replication and encapsidation of recombinant adenoviral DNA into infectious virions and the formation of plaques. Individual plaques were isolated and amplified in 293 cells, viral DNA was isolated (Hirt, 1967), and recombinant adenovirus plaques containing the human CFTR cDNA (Ad-CFTR) were identified by restriction cleavage and Southern analysis. Ad-CFTR and the control viruses Ad-α1AT (Rosenfeld et al., 1991a) and the E1a deletion virus Ad-dl312 (Jones and Shenk, 1979) were propagated in 293 cells and recovered 36 hr after infection by 5 cycles of freeze/thawing. All viral preparations were purified by CsCl density centrifugation (Graham and Van Der Eb, 1973), dialyzed, and stored in virus dialysis buffer (10 mM Tris–HCl [pH 7.4], 1 mM $MgCl_2$) at 4°C for immediate use or, with the addition of 10% glycerol, at −70°C prior to use. Titers of the viral stocks were determined by plaque assay using 293 cells (Graham and Van Der Eb, 1973; Graham et al., 1977).

In Vitro and In Vivo Infection with Ad-CFTR

For in vitro infection, 293 or CFPAC-1 cells were trypsinized, counted, and seeded (10 cm plates [4.5 × 10^6 cells per plate] for evaluation of CFTR mRNA or synthesis of CFTR protein; 6 cm plates [5.0 × 10^5 cells per plate] for evaluation of Cl^- permeability in response to forskolin) and infected with Ad-CFTR, Ad-dl312, or Ad-α1AT (200 plaque-forming units [PFU] per cell for CFPAC-1 cells; 50 PFU per cell for 293 cells). After 18–24 hr (for 293 cells) or 48 hr (for CFPAC-1 cells), cells were evaluated for the presence of CFTR mRNA, synthesis of CFTR protein, and Cl^- permeability in response to forskolin (see below). For in vitro infection of respiratory epithelium, cotton rats (Sigmodon hispidus) were sacrificed and the lungs and trachea were isolated by a midline thoracic incision. Cells were obtained by cytologic brush and infected as previously described (Rosenfeld et al., 1991a). For in vivo studies, cotton rats were anesthetized by methoxyfluorane inhalation. The trachea was exposed by anterior midline incision, and 10^{10} to 5 × 10^{11} PFU of Ad-CFTR or Ad-dl312 was instilled into the trachea in a total volume of 300 μl. For virus stored at −70°C, the diluent was phosphate-buffered saline (PBS) (pH 7.4). An equal volume of PBS (with glycerol at the same final concentration contained in the diluted virus used for the infection) was instilled in other cotton rats as an additional negative control. Virus used immediately after dialysis was not diluted, and thus, corresponding control animals received an equal volume of the virus dialysis buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$.

Northern Analysis of CFTR mRNA Transcripts

For in vitro infection experiments, total RNA was isolated from 293 cells or CFPAC-1 cells (at 20 and 48 hr after adenoviral vector infection, respectively) or as a control, from uninfected subconfluent T84 cells, using the guanidine thiocyanate–CsCl technique (Chirgwin et al., 1979). For in vivo studies, cotton rats were evaluated 2 days to 6 weeks after infection and the lungs were isolated as described above. Following exsanguination by cardiac puncture, the lungs were lavaged twice, the pulmonary artery was perfused with PBS, the lungs and trachea were resected and minced, and total lung RNA was extracted (Chirgwin et al., 1979; Rosenfeld et al., 1991a).

RNA was subjected to formaldehyde–agarose gel electrophoresis, transferred to a nylon membrane (Nytran, Schleicher & Schuell), hybridized with a ^{32}P-labeled, 4.5-kb CFTR cDNA probe prepared by random [illegible] as previously described (Yoshimura et al., 1991a). As a control, the same membrane was subsequently hybridized with either a human β-actin cDNA probe (pHFβA-1; Gunning et al., 1983) for cultured human cells or a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Tso et al., 1985) for rat lung RNA. The relative amount of human CFTR mRNA at 6 weeks compared to 2 days was quantified by laser densitometry.

In Vitro Evaluation of the Function of Ad-CFTR-Derived Human CFTR

In vitro synthesis of human CFTR was evaluated in 293 and CFPAC-1 cells after Ad-CFTR infection. Cells were incubated in labeling medium (methionine-free medium containing [^{35}S]methionine (500 μCi/ml, 1000 Ci/mmol, New England Nuclear] during the entire infection period. After infection (24 hr for 293 cells; 48 hr for CFPAC-1 cells), cells were washed twice in PBS and solubilized at 4°C in lysis buffer (PBS containing 10 mM ethylenediaminetetraacetate, 1% Triton X-100, 0.5% sodium deoxycholate, 200 μg/ml aprotinin, 100 μg/ml leupeptin [both from Boehringer Mannheim], and 2 mM phenymethylsulfonyl fluoride [PMSF, Sigma]). The cell lysate was frozen (−70°C for at least 30 min), thawed, and clarified by centrifugation (12,000 g, 4°C, 30 min). Human CFTR synthesis was then evaluated by immunoprecipitation of ^{35}S-labeled human CFTR, SDS–polyacrylamide gel electrophoresis, and fluorography (Mornex et al., 1986; Rosenfeld et al., 1991a). To accomplish this, equal amounts of total trichloroacetic acid precipitable radioactivity (3 × 10^6 dpm for all 293 cell samples, 30 × 10^6 dpm for all CFPAC-1 cell samples) were immunoprecipitated using a mouse anti-human CFTR monoclonal antibody (Genzyme).

To demonstrate that the Ad-CFTR vector was capable of directing the expression of functional CFTR protein, forskolin-stimulated Cl^- permeability was evaluated in cells that do not normally exhibit cAMP-mediated Cl^- permeability (293 cells) or in human epithelial cells derived from an individual with CF (CFPAC-1 cells, a cell line with homozygous expression of the ΔF508 CF mutation [Schoumacher et al., 1990]). To accomplish this, at 18 hr (293 cells) or 48 hr (CFPAC-1 cells) after infection with Ad-CFTR, Cl^- efflux was evaluated (Trapnell et al., 1991a). Briefly, monolayers of 293 or CFPAC-1 cells were washed twice with Ringer's lactate and loaded with $^{36}Cl^-$ (2.5 μCi/ml; >3 mCi per gram of Cl^- [Amersham]; 2 hr, 37°C) and then washed rapidly with 3 ml aliquots (× 6) of Ringer's lactate buffer. $^{36}Cl^-$ efflux was measured by sequentially removing and replacing buffer (0.9 ml aliquots) at various time intervals for up to 7.5 min. The cells were then removed from the dish with 0.9 ml of 0.25% trypsin (Biofluids), and the amount of $^{36}Cl^-$ in the efflux aliquots and cells was determined by liquid scintillation counting. The total radioactivity loaded into cells was calculated from the sum of the individual efflux time points plus that remaining in cells at the end of the sampling period, and the Cl^- efflux data were plotted for each time point as $^{36}Cl^-$ remaining in cells as a percentage of total $^{36}Cl^-$ initially loaded into cells. Forskolin-stimulated Cl^- permeability was evaluated by adding 13 μM forskolin (Sigma) to the sampling buffer used for collection of the efflux samples.

Analysis of CFTR mRNA Expression by In Situ Hybridization

Cotton rat lungs and trachea were isolated as described above. After blood was removed by cardiac puncture, the lungs were fixed with 4% paraformaldehyde (PFA, Fluka) infused into the trachea and pulmonary artery, and cryostat sections (7–10 μm) were prepared and stored frozen (−70°C) until use. Immediately prior to hybridization, cryostat sections were sequentially treated with 0.2 M HCl and 1 μg/ml proteinase K. Three different sets of human CFTR ^{35}S-labeled sense and antisense cRNA probes were synthesized in vitro from plasmid transcription vectors (pGEM, Promega) with [^{35}S]UTP (1 mCi, 800 Ci/mmol, SP6/T7 grade; Amersham) by standard techniques. Each pGEM CFTR vector contained a different region of human CFTR cDNA (exons 1–5, 9–13, or 21–24). The antisense and sense probes were combined, respectively, and hydrolyzed, and the lung tissue sections were then hybridized (12 hr, 50°C) with the labeled cRNA probes (1.2 × 10^5 dpm/μl). Lung tissue sections were then washed, treated with RNAase A (50 μg/ml, Sigma), washed, dehydrated, and evaluated by autoradiography (10 days) and counterstained with hematoxylin and eosin (Harper et al., 1985; Bernaudin et al., 1988; Rosenfeld et al., 1991a). Relative expression of human CFTR mRNA in epithelium versus submucosa was determined by calculating the mean number of silver [illegible] and subtracting the mean background grains from

each (defined as number of silver grains in cell-free areas), and expressing this value as a ratio of expression in epithelium versus subepithelium.

Detection of CFTR mRNA Transcripts Using the Polymerase Chain Reaction

Ad-CFTR-directed CFTR mRNA transcripts were evaluated in rat lung RNA (prepared as above) after conversion to cDNA, PCR amplification, and Southern hybridization analysis. RNA was first treated with DNAase (10 U per μg of RNA; RNAase-free RQ1 DNAase, Promega) to eliminate possible residual viral DNA. RNA was then converted to cDNA by standard techniques using Moloney murine leukemia virus reverse transcriptase with random hexanucleotide primers (Roth et al., 1985) and amplified by PCR (25 cycles) and Taq DNA polymerase (Perkin Elmer Cetus [Saiki et al., 1988]). To ensure that Ad-CFTR-driven transcripts were specifically evaluated and that the 5' and 3' portions of the mRNA transcripts were present, two separate primer pairs were used: a 5' primer pair to detect the 5' end of Ad-CFTR mRNA transcripts consisting of an adenoviral-specific sense primer in the tripartite leader sequence (VAD-5; 5'-AGCTGTTGGGGCTCGCGGTTGAGG-3') and a human CFTR-specific antisense primer in CFTR exon 5 (HCF60; 5'-CATCAAATTTGTTCAGGTTGTTGG-3'), and a 3' primer pair to evaluate the 3' end of Ad-CFTR mRNA transcripts consisting of a human CFTR-specific sense primer in CFTR exon 21 (HCF12; 5'-AGTGGAGTGATCAAGAAATATGG-3') and an SV40 viral-specific primer in the SV40 early mRNA polyadenylation signal sequence (SVPOLYA; 5'-GTAACCATTATAAGCTGCAATAAAC-3' [Fiers et al., 1978]). As a control, rat GAPDH transcripts corresponding to amino acid residues 126–300 were amplified under similar conditions using GAPDH transcript-specific primers (GAPDH-1; 5'-AATGCATCCTGCACCACCAACTGC-3' and GAPDH-2; 5'-GGAGGCCATGTAGGCCATGAGGTC-3' [Tso et al., 1985]). Each DNAase-treated RNA sample was also used as a PCR template in parallel without conversion to cDNA, to eliminate the possibility that amplification of potentially contaminating viral DNA occurred. PCR amplification products were evaluated by agarose gel electrophoresis followed by Southern hybridization using nested or internal ^{32}P-labeled human CFTR cDNA probes (a 462 bp PvuII–XbaI fragment spanning exons 2–5 [Riordan et al., 1989] for the 5' region amplification products, or a 200 bp fragment spanning exons 22–23 [Riordan et al., 1989; Yoshimura et al., 1991a; Trapnell et al., 1991a] for the 3' region amplification products) or an internal rat GAPDH cDNA probe (a 281 bp BanII fragment within the amplified GAPDH transcript region described above [Tso et al., 1985]).

Immunohistochemical Detection of the Human CFTR Protein after In Vivo Infection with Ad-CFTR

Human CFTR was evaluated in cytocentrifuge preparations of cotton rat lung epithelium infected in vitro or obtained from cotton rat lung 11–14 days after in vivo Ad-CFTR intratracheal instillation using the alkaline phosphatase monoclonal anti-alkaline phosphatase (APAAP) method (Cordell et al., 1984) and anti-human CFTR antibody (Genzyme) with minor modifications. Briefly, for in vitro studies, cells were collected 48 hr after in vitro infection with the recombinant adenovirus and resuscended in Hanks balanced salt solution (HBSS, Mediatech) supplemented with 2% heat-inactivated fetal calf serum (Biofluids), and cytopreps slides were prepared by cytocentrifugation (450 rpm, 3 min). For the in vivo studies, the airway epithelial cells were obtained from cotton rats as described above and immediately cytocentrifuged. As controls, T84 cells were trypsinized and prepared in the same manner. Air-dried slides were evaluated immediately or stored at −70°C until evaluation. Cytopreps were fixed in acetone for 10 min at −20°C and air-dried. Cells were first incubated with a mouse anti-human CFTR monoclonal antibody raised against a CFTR exon 13-β-galactosidase fusion protein (1 μg/ml, Genzyme) in the presence of protease inhibitors (100 μg/ml aprotinin, 50 μg/ml leupeptin, 15 μg/ml chymostatin, 0.575 mM phenylmethylsulfonyl fluoride [all from Boehringer Mannheim], 7.5 μg/ml pepstatin [Cal Biochem], 1.5 mM p-aminobenzoic acid [Fluka]). Slides were subsequently incubated with rabbit anti-mouse immunoglobulin (96 μg/ml, Dako), followed by alkaline phosphatase mouse monoclonal anti-alkaline phosphatase (8.3 μg/ml, Dako). The intensity of immunoreactivity was enhanced by repeating the last two antibody reaction steps. Immunoreactivity was detected using a fuchsin substrate-chromogen technique (Histo-Mark™ Red test system, Kirkgaard & Perry). As controls, duplicate slides were evaluated in the absence of the primary anti-human CFTR antibody or with an irrelevant primary mouse monoclonal antibody (anti-human Leu-2A (CD8), Becton Dickinson).

Acknowledgments

We thank R. Frizzell (Department of Physiology, University of Alabama) for the generous gift of CFPAC-1 cells; T. Banks and D. Dreyer for technical assistance; S. Rosenfeld (Laboratory of Clinical Hematology, National Heart, Lung and Blood Institute), C. Danel, and R. Wersto for assistance with immunohistochemistry; D. Villeval for sequence analysis; R. Dreyfuss for help with photomicroscopy; G. Prince and M. Reddington (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases) for making cotton rats available to us; T. Shenk for the gift of Ad-dl312; and T. Raymer for editorial assistance. This work was supported in part by the American Cystic Fibrosis Foundation and l'Association Française de lutte contre la Mucoviscidose. W. G. was supported by the Cystic Fibrosis Foundation Research Development Program and NIH grants HL 40178 and HL 47122.

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Received August 5, 1991; revised November 15, 1991.

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